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Intracellular Siderophores Are Essential for Ascomycete Sexual Development in Heterothallic *Cochliobolus heterostrophus* and Homothallic *Gibberella zeae*▽†

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Connections between fungal development and secondary metabolism have been reported previously, but as yet, no comprehensive analysis of a family of secondary metabolites and their possible role in fungal development has been reported. In the present study, mutant strains of the heterothallic ascomycete *Cochliobolus heterostrophus*, each lacking one of 12 genes (*NPS1* to *NPS12*) encoding a nonribosomal peptide synthetase (NRPS), were examined for a role in sexual development. One type of strain (Δ*nps2*) was defective in ascus/ascospore development in homozygous Δ*nps2* crosses. Homozygous crosses of the remaining 11 Δ*nps* strains showed wild-type (WT) fertility. Phylogenetic, expression, and biochemical analyses demonstrated that the NRPS encoded by *NPS2* is responsible for the biosynthesis of ferricrocin, the intracellular siderophore of *C. heterostrophus*. Functional conservation of *NPS2* in both heterothallic *C. heterostrophus* and the unrelated homothallic ascomycete *Gibberella zeae* was demonstrated. *G. zeae* Δ*nps2* strains are concomitantly defective in intracellular siderophore (ferricrocin) biosynthesis and sexual development. Exogenous application of iron partially restored fertility to *C. heterostrophus* and *G. zeae* Δ*nps2* strains, demonstrating that abnormal sexual development of Δ*nps2* strains is at least partly due to their iron deficiency. Exogenous application of the natural siderophore ferricrocin to *C. heterostrophus* and *G. zeae* Δ*nps2* strains restored WT fertility. *NPS1*, a *G. zeae NPS* gene that groups phylogenetically with *NPS2*, does not play a role in sexual development. Overall, these data demonstrate that iron and intracellular siderophores are essential for successful sexual development of the heterothallic ascomycete *C. heterostrophus* and the homothallic ascomycete *G. zeae*.

The innate benefits, to their producers, of the preponderance of diverse metabolites biosynthesized by fungal and bacterial nonribosomal peptide synthetases (NRPSs) are largely unknown. In contrast, because of the medicinal, pharmaceutical, or industrial value of some NRPS metabolites, considerable effort has been expended in characterizing these metabolites with respect to their effects (e.g., antibiotic, immunosuppressive) on other organisms, particularly humans.

Our objective is to determine the natural biological functions of NRPS metabolites in the organisms that produce them; for this, we focus on the genetically tractable heterothallic ascomycete pathogen of maize *Cochliobolus heterostrophus*. Twelve genes that encode NRPSs (*NPS1* to *NPS12*) have been identified in the *C. heterostrophus* genome (18, 23). Initially, we focused on the role of NRPS metabolites in virulence to the plant host, since the best-known NRPS metabolites in phytopathogenic ascomycetes are phytotoxins, such as AM-toxin, made by the apple pathotype of *Alternaria alternata*, and HC-toxin, made by race 1 of *Cochliobolus carbonum*, both crucial for the pathogenicity of the producing organisms to their hosts. Characterization of *C. heterostrophus* mutant strains carrying a single-gene deletion of each of the 12 *NPS* genes, with respect to their virulence to maize, revealed that only *NPS6* is required for this activity (18, 23). Further characterization revealed that Δ*nps6* strains were sensitive also to oxidative (18) and low-iron (23) stress, phenotypes that, in combination, led to the recognition that the product of the NPS6 protein was a siderophore. Mass spectrometry (MS) and high-performance liquid chromatography (HPLC) analyses identified the siderophore products of the NPS6 protein as coprogen and derivatives, extracellular siderophores, demonstrating that extracellular siderophores play a role in fungal virulence to plant hosts (23).

Although iron is an essential nutrient for virtually all organisms, including fungi, bioavailable forms are scarce in aerobic environments because of oxidation and formation of minimally soluble iron hydroxides (19). When starved for iron, many fungi and bacteria secrete siderophores to acquire iron extracellularly. The strong iron-chelating activity of these low-molecular-weight organic compounds can solubilize iron effectively from the environment (25, 35). Indeed, as noted above, preventing extracellular siderophore biosynthesis by deletion of *NPS6* in *C. heterostrophus* leads to hypersensitivity to iron depletion, which highlights the major contribution of siderophores to iron gathering in this species (23). Similarly, Δ*nps6* strains of two other filamentous ascomycetes, a second cereal pathogen, *Gibberella zeae*/*Fusarium graminearum*, and the dicot pathogen *Alternaria brassicicola*, lacking the ability to biosynthesize extracellular siderophores, are hypersensitive to

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iron depletion and reduced in virulence to their hosts, indicating that siderophore-mediated iron metabolism is generally important among filamentous ascomycete phytopathogens (23). Except for the polycarboxylates identified in zygomycetes (31), all fungal siderophores are products of NRPSs.

While characterizing the role of *C. heterostrophus NPS6* in extracellular siderophore biosynthesis, we identified ferricrocin as the intracellular siderophore of this species and determined also that the NRPS encoded by *NPS6* is not involved in its biosynthesis (i.e., the Δ*nps6* strain produces wild-type [WT] levels of ferricrocin) (23). Intracellular siderophores, in contrast to extracellular ones, have been proposed to play a role in iron storage in mycelia and spores (36) and have been recognized as asexual spore germination factors of *Neurospora crassa*, *Penicillium chrysogenum*, and *Aspergillus nidulans* (9, 15). In these species, treatment of asexual spores with solutions with low water activity (high salinity) led to loss of intracellular siderophores stored in the spores and to inhibition or delay of spore germination. In addition, *A. nidulans* intracellular siderophores are essential for efficient asexual sporulation (12). In contrast, deletion of *C. heterostrophus NPS6*, which is responsible for extracellular siderophore production, leads to a reduction of asexual sporulation, as well as to a reduction of virulence to the host (23). Recently, intracellular siderophores have been reported also to play a role in the self-compatible sexual development of *A. nidulans* (13). In addition to siderophores, other small molecules, such as the oxylipin psi factors, have been demonstrated to alter the ratio of asexual to sexual sporulation in *A. nidulans* (5, 6, 33) and a small γ-butyrolactone-containing molecule, butyrolactone I, has been reported as an inducer of asexual sporulation in *A. nidulans* (27). This close relationship between secondary-metabolite or small-molecule production and fungal development has been documented comprehensively for *Aspergillus* spp. (reviewed in references 7 and 37). It has been demonstrated that sporulation and sterigmatocystin-aflatoxin production are regulated by a common G-protein/cyclic-AMP/protein kinase A signaling pathway (7).

The primary objective of the present study was to determine if one or more *C. heterostrophus* NRPS metabolites function in sexual development, by mating assay screening of our collection of mutant strains, each with 1 of the 12 *NPS* genes deleted. We found that *NPS2* is involved in ascospore development. Δ*nps2* strains fail to form asci and ascospores in crosses in which the Δ*nps2* mutation is homozygous; otherwise, sexual development is normal up to this stage. Through biochemical analyses of WT and Δ*nps2* strains, we identified the product of the NRPS2 enzyme as the intracellular siderophore ferricrocin, thus connecting the production of this small molecule to a fundamental fungal development process. Furthermore, we demonstrated functional conservation of NPS2 in ferricrocin biosynthesis and in sexual development in the heterothallic ascomycete *C. heterostrophus* and in the unrelated homothallic ascomycete *G. zeae*. We propose that iron and intracellular siderophores are essential for successful sexual development in both self-incompatible and self-compatible ascomycetes.

MATERIALS AND METHODS

Strains and culture conditions. *C. heterostrophus* WT strains C4 (*MAT1-2*; ATCC 48331) and CB7 (*MAT1-1 alb1*; ATCC 48332) and *G. zeae* WT strain Gz3639 (NRRL 29169) were used as background strains for all experiments. Unless otherwise mentioned, all cultures were grown on complete medium (CM) (17) at 24°C under continuous fluorescent light illumination. Transgenic strains developed in this study or in the study of Oide et al. (23) and their progeny are listed in Table 1.

Gene identification. The 12 *C. heterostrophus NPS* genes (accession no. AY884186 to AY884197) were annotated as described previously (18, 23). Phylogenetic analysis (see Fig. 7 in reference 18; K. E. Bushley and B. G. Turgeon, unpublished data) indicates that *C. heterostrophus* NPS2 falls in a group that includes *NPS* genes that encode NRPSs known to produce siderophores. These include *A. nidulans* SidC (accession no. AAP56239/AN0607.3) and *Ustilago maydis* Sid2 (accession no. AAB93493/UMU62738).

The *G. zeae NPS2* and *NPS1* genes were identified by BLAST query of the *G. zeae* genome sequence (http://mips.gsf.de/genre/proj/fusarium/) with the four AMP-binding domains that make up the *C. heterostrophus* NPS2 protein (18) and with AMP-binding domains of *A. nidulans* SidC and *U. maydis* Sid2. The top *G. zeae* hits were extracted and annotated, and all of the AMP-binding domains were used for phylogenetic analysis as in Fig. 7 of reference 18 (data not shown).

DNA manipulations. Fungal genomic DNA was prepared as described previously (11, 23). Unless otherwise mentioned, all PCRs were performed with PCR master mix (Promega) by following the manufacturer's recommendations.

Deletion of the complete set of *C. heterostrophus NPS* genes. For targeted gene deletion, split marker constructs were prepared (see Table S1 in the supplemental material) as previously described (8, 23). In previous work on *NPS* genes (18), partial gene deletions were generated for 11 of the *NPS* genes. *NPS12* was not included. For the present study, the open reading frame (ORF) of each of genes *NPS1* to *-3* and *NPS5* to *-12* was completely deleted and 94% of that of *NPS4* was deleted (see Table S1 in the supplemental material) by replacement with the *hygB* cassette from pUCATPH (20), which confers resistance to hygromycin B. Transformation of *C. heterostrophus* was carried out as described previously (23).

Deletion of *G. zeae NPS1* and *NPS2* genes. We deleted 94% of the *G. zeae NPS1* and *NPS2* ORFs (see Table S1 in the supplemental material) and replaced the deleted portions with the *hygB* gene cassette from pUCATPH. *G. zeae* Δ*nps1* Δ*nps2* strains were constructed from strain Fgnps2-6-1 (Δ*nps2 hygB*^{R}) (Table 1), which carries a deletion of *NPS2*. The *NPS1* ORF was replaced with the *nptII* gene (23), which confers resistance to G418. Transformation of *G. zeae* was carried out as described by Oide et al. (23).

Complementation. Reintroduction of the WT *C. heterostrophus NPS2* ORF was carried out with strain Chnps2-3p (Table 1), which carries a deletion of the first thiolation domain of the Ch*NPS2* ORF, as described by Lee et al. (18). The construct for reintroduction of the *C. heterostrophus NPS2* ORF was prepared by PCR with genomic DNA and Platinum *Taq* High Fidelity (Invitrogen) by following the manufacturer's directions. A 2,241-bp DNA fragment including the deleted region (342 bp) and the surrounding flanking sequences (829-bp 5′flank and 1,070-bp 3′flank) was amplified. Strain Chnps2-3p was transformed with approximately 5 μg of the PCR product and 10 μg of plasmid pII99 (23) carrying the *nptII* gene. Initial screening of transformants was carried out by overlaying a 1% agar solution containing 600 μg/ml G418 on the transformation plates. Secondary screening of transformants was carried out on CM without salts (23) containing 400 μg G418/ml. Transformants resistant to G418 were selected and screened for sensitivity to hygromycin B. Replacement of the *hygB* gene in G418^R hygB^S transformants, at the *Nps2* locus, with the *NPS2* fragment was confirmed by PCR as described previously for *NPS6* (23).

Crosses. For *C. heterostrophus*, crosses were set up as described previously (17), except that minimal medium (MM) without glucose (MMNOS) was used instead of Sach's medium (21). MMNOS is easier to prepare than Sach's medium, and to date, no differences in the fertility of any cross tested have been observed when pseudothecia from each medium type were compared.

For *G. zeae*, crosses were set up as described previously (10) except that carrot medium was replaced with carrot juice (CJ) medium. In our experiments, WT *G. zeae* showed better fertility on CJ medium than on medium made with fresh carrots. CJ medium is 50 ml of 100% carrot juice (LakeWood, Miami, FL), 3.0 g of $CaCO_3$, and 950 ml of H_2O per liter of medium. For mating, growing mycelial tips were placed in the centers of CJ agar plates and the plates were sealed with Parafilm and incubated under continuous black light at 24°C for 7 days. Approximately 1 ml of sterile 2.5% Tween 60 solution was applied to the plate, and mycelia growing on the plate were knocked down with a rubber policeman, which induced sexual development. Excess Tween 60 solution was discarded and the unsealed plates were incubated under the original conditions for an additional 7 days.

Screening of all *C. heterostrophus* Δ*nps* strains for a role in sexual development. (i) Crosses of *C. heterostrophus* Δ*nps* strains to the WT. The complete set of 12 Δ*nps* strains was screened for a role in sexual development. For all exper-

TABLE 1. Strains used in this study

Species and strain[a]	Genotype	Genetic background or reference
C. heterostrophus		
C4	*MAT1-2*	17
CB7	*MAT1-1*	4
Chnps1-1	Δ*nps1 MAT1-2 hygB*	C4
Chnps1-2	Δ*nps1 MAT1-2 hygB*	C4
Chnps1-8	Δ*nps1 MAT1-2 hygB*	C4
1447-T1-1	Δ*nps1 MAT1-1 hygB*	Progeny of Chnps1-1 × CB7
1448-T1-2	Δ*nps1 MAT1-1 hygB*	Progeny of Chnps1-2 × CB7
1448-T1-6	Δ*nps1 MAT1-2 hygB alb1*	Progeny of Chnps1-2 × CB7
Chnps2-1	Δ*nps2 MAT1-2 hygB*	C4
Chnps2-2	Δ*nps2 MAT1-2 hygB*	C4
Chnps2-3p[b]	Δ*nps2 MAT1-2 hygB*	C4
1449-T1-5	Δ*nps2 MAT1-1 hygB alb1*	Progeny of Chnps2-1 × CB7
1449-T1-1	Δ*nps2 MAT1-2 hygB*	Progeny of Chnps2-1 × CB7
1451-T1-2	Δ*nps2 MAT1-1 hygB*	Progeny of Chnps2-2 × CB7
1451-T1-3	Δ*nps2 MAT1-2 hygB*	Progeny of Chnps2-2 × CB7
Chnps3-3	Δ*nps3 MAT1-2 hygB*	C4
Chnps3-4	Δ*nps3 MAT1-2 hygB*	C4
1437-T1-2	Δ*nps3 MAT1-2 hygB alb1*	Progeny of Chnps3-3 × CB7
1437-T1-6	Δ*nps3 MAT1-1 hygB*	Progeny of Chnps3-3 × CB7
1438-T1-2	Δ*nps3 MAT1-2 hygB*	Progeny of Chnps3-4 × CB7
1438-T1-8	Δ*nps3 MAT1-1 hygB alb1*	Progeny of Chnps3-4 × CB7
Chnps4-3	Δ*nps4 MAT1-2 hygB*	C4
Chnps4-4	Δ*nps4 MAT1-2 hygB*	C4
1466-T1-2	Δ*nps4 MAT1-1 hygB*	Progeny of Chnps4-4 × CB7
1466-T1-6	Δ*nps4 MAT1-2 hygB alb1*	Progeny of Chnps4-4 × CB7
1466-T1-7	Δ*nps4 MAT1-1 hygB*	Progeny of Chnps4-4 × CB7
1466-T1-8	Δ*nps4 MAT1-2 hygB alb1*	Progeny of Chnps4-4 × CB7
Chnps5-1	Δ*nps5 MAT1-2 hygB*	C4
Chnps5-4	Δ*nps5 MAT1-2 hygB*	C4
Chnps5-5	Δ*nps5 MAT1-2 hygB*	C4
1443-T1-3	Δ*nps5 MAT1-1 hygB*	Progeny of Chnps5-5 × CB7
1443-T1-7	Δ*nps5 MAT1-1 hygB*	Progeny of Chnps5-5 × CB7
Chnps6-1[c]	Δ*nps6 MAT1-2 hygB*	C4
Chnps6-2[c]	Δ*nps6 MAT1-2 hygB*	C4
Chnps6-1R2[c]	Δ*nps6 MAT1-1 hygB*	Progeny of Chnps6-1 × CB7
Chnps7-2	Δ*nps7 MAT1-2 hygB*	C4
Chnps7-5	Δ*nps7 MAT1-2 hygB*	C4
1435-T1-2	Δ*nps7 MAT1-1 hygB alb1*	Progeny of Chnps7-2 × CB7
1436-T1-1	Δ*nps7 MAT1-1 hygB alb1*	Progeny of Chnps7-5 × CB7
Chnps8-1	Δ*nps8 MAT1-2 hygB*	C4
Chnps8-4	Δ*nps8 MAT1-2 hygB*	C4
1468-T1-2	Δ*nps8 MAT1-1 hygB alb1*	Progeny of Chnps8-4 × CB7
1468-T1-3	Δ*nps8 MAT1-1 hygB alb1*	Progeny of Chnps8-4 × CB7
Chnps9-1	Δ*nps9 MAT1-2 hygB*	C4
Chnps9-3	Δ*nps9 MAT1-2 hygB*	C4
1530-T1-8	Δ*nps9 MAT1-1 hygB alb1*	Progeny of Chnps9-1 × CB7
Chnps10-2	Δ*nps10 MAT1-2 hygB*	C4
Chnps10-5	Δ*nps10 MAT1-2 hygB*	C4
1479-T1-4	Δ*nps10 MAT1-1 hygB*	Progeny of Chnps10-2 × CB7
1480-T1-6	Δ*nps10 MAT1-2 hygB*	Progeny of Chnps10-5 × CB7
1480-T1-8	Δ*nps10 MAT1-2 hygB alb1*	Progeny of Chnps10-5 × CB7
Chnps11-5	Δ*nps11 MAT1-2 hygB*	C4
Chnps11-7	Δ*nps11 MAT1-2 hygB*	C4
1450-T1-2	Δ*nps11 MAT1-1 hygB alb1*	Progeny of Chnps11-7 × CB7
1450-T1-3	Δ*nps11 MAT1-2 hygB*	Progeny of Chnps11-7 × CB7
1452-T1-1	Δ*nps11 MAT1-1 hygB*	Progeny of Chnps11-7 × CB7
1452-T1-2	Δ*nps11 MAT1-2 hygB alb1*	Progeny of Chnps11-7 × CB7
Chnps12-1	Δ*nps12 MAT1-2 hygB*	C4
Chnps12-3	Δ*nps12 MAT1-2 hygB*	C4
1550-T1-1[d]	Δ*nps12 MAT1-1 hygB alb1*	Progeny of Chnps12-1 × 1450-T1-2
G. zeae		
Gz3639	WT	2
Fgnps1-1-1[a]	Δ*nps1 hygB*	Gz3639
Fgnps1-5-1	Δ*nps1 hygB*	Gz3639
Fgnps2-5-1	Δ*nps2 hygB*	Gz3639
Fgnps2-6-1	Δ*nps2 hygB*	Gz3639
Fgnps2-6-1Δnps1-2-1[a]	Δ*nps1* Δ*nps2 hygB nptII*	Fgnps2-6-1
Fgnps2-6-1Δnps1-5-1	Δ*nps1* Δ*nps2 hygB nptII*	Fgnps2-6-1

[a] Nomenclature: Chnps1-1 is a strain in which *NPS1* is deleted; -1 indicates transformant 1; 1447-T1-1 is cross no. 1447; T1-1 is tetrad no. 1, ascospore 1; for Fgnps2-6-1, nps2 refers to deletion of *NPS2*; 6 refers to transformant no. 6, and 1 refers to purified conidial isolate 1 of transformant 6; Fgnps2-6-1Δnps1-2-1 is single conidial isolate 1 of transformant 2 of Fgnps2-6-1Δnps1-2, in which the *NPS1* gene was deleted in strain Fgnps2-6-1.

[b] Only a portion of the *NPS2* ORF is deleted in this strain.

[c] See reference 23.

[d] This strain was obtained as a derivative of a cross aimed to isolate Δ*nps11* Δ*nps12*, which will be reported in the future.

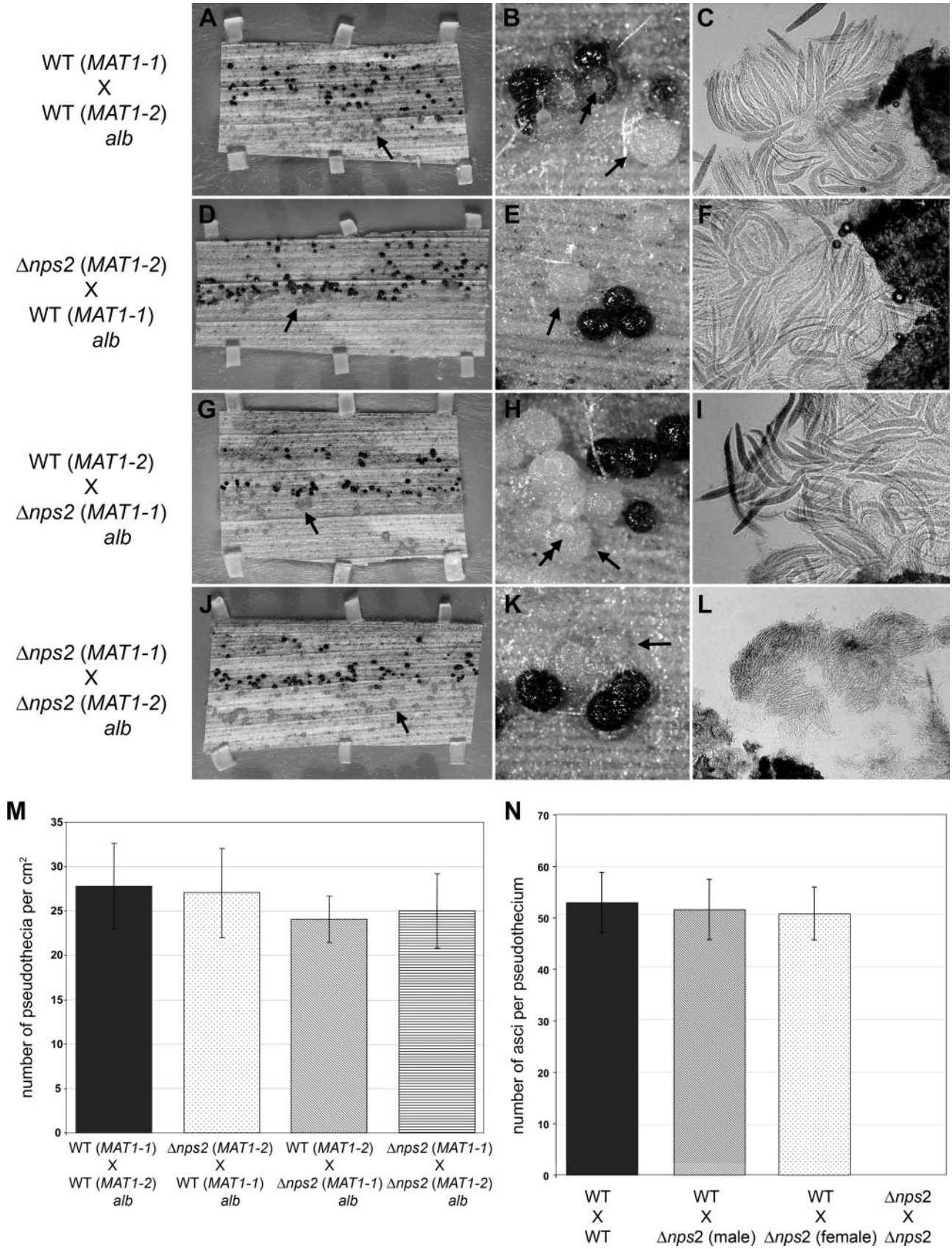


FIG. 1. *C. heterostrophus* Δ*nps2* strains form pseudothecia as well as WT strains do, but homozygous Δ*nps2* crosses fail to develop asci and ascospores. (A, B, D, E, G, H, J, K) Pseudothecia on senescent corn leaves 21 days after cross setup. Images A, D, G, and J were taken at ×6.4

iments, crosses between *C. heterostrophus* strains C4 (*MAT1-2*) and CB7 (*MAT1-1 alb1*) were set up as WT controls. The fertility of Δ*nps* strains in heterozygous crosses was evaluated by crossing each of the 12 original Δ*nps* strain (all *MAT1-2*, pigmented) to tester strain CB7 (*MAT1-1 alb1*). At least two independent transformants were examined for each of the 12 *NPS* genes deleted, and each cross combination was duplicated. The ability of strains to act both male and female (as hermaphrodites) was tracked by crossing pigmented Δ*nps* strains to albino WT testers of the opposite mating type. If a Δ*nps* strain retains WT hermaphroditic ability, both pigmented and albino pseudothecia should form, as pseudothecium color is determined by the female partner. Thus, the ability of the pigmented Δ*nps* strains to act female was assessed by monitoring for the presence or absence of fertile pigmented pseudothecia, while the ability of each Δ*nps* strain to act male was assessed by monitoring for the presence or absence of fertile albino pseudothecia.

The number of pseudothecia per square centimeter of corn leaf was recorded for each cross. For the initial screening, at least 10 pigmented and 10 albino pseudothecia were opened and the number of asci in each pseudothecium was recorded.

(ii) *C. heterostrophus* Δ*nps* × Δ*nps* strain crosses. The fertility of *C. heterostrophus* Δ*nps* strains in homozygous crosses was evaluated in essentially the same way as described for heterozygous crosses. *C. heterostrophus* Δ*nps MAT1-1* strains were obtained as follows. $hygB^R$ progeny were recovered from a cross between the original Δ*nps MAT1-2* transformants and CB7 (*MAT1-1 alb1*), and their mating type was determined by crossing them to *MAT1-2* (strain C4) and *MAT1-1* (strain CB7) testers. For each homozygous Δ*nps* cross, at least two different combinations of independent Δ*nps* strains were set up (e.g., for ChΔ*nps2* × ChΔ*nps2*, 1449-T1-1 × 1449-T1-5 and 1451-T1-2 × 1451-T1-3 were examined; see Table S2 in the supplemental material and Table 1). Each cross was duplicated.

Evaluation of the fertility of *C. heterostrophus* Δ*nps2* strains and *G. zeae* Δ*nps1*, Δ*nps2*, and Δ*nps1* Δ*nps2* strains. (i) *C. heterostrophus*. After the initial analyses, the fertility of Δ*nps2* strains was examined more extensively. The fertility of Δ*nps2* strains in a heterozygous cross was examined also in a *MAT1-1* background in order to exclude the possibility that the observed abnormal sexual development was connected in some way to the mating type. Note that only Δ*nps2* strains with *MAT1-2* backgrounds were examined in the initial characterization of fertility in a heterozygous cross.

Ten replicates of each WT × WT, WT × Δ*nps2 MAT1-1*, WT × Δ*nps2 MAT1-2*, and Δ*nps2 MAT1-1* × Δ*nps2 MAT1-2* cross were set up, and the number and color of pseudothecia per square centimeter of leaf were recorded for each cross. The data were analyzed by analysis of variance (ANOVA). More than 30 pseudothecia were opened for each type of cross, and the number of asci in each pseudothecium was recorded. The data were statistically evaluated by ANOVA (note that no asci are formed in Δ*nps2* × Δ*nps2*; hence, the data were excluded from the statistical test).

(ii) *G. zeae*. For homothallic *G. zeae*, each mutant strain was selfed and its fertility was evaluated by counting the perithecia on each plate and determining the number of asci per perithecium. A selfed WT strain was set up for all experiments as a control. For evaluation of perithecium formation, 10 plates were set up for each genotypic class (i.e., Δ*nps1*, Δ*nps2*, Δ*nps1* Δ*nps2*, and WT) and the number of perithecia formed on each plate was recorded. Data were analyzed by ANOVA. At least 30 perithecia were opened for each genotypic class, and the number of asci was recorded for each perithecium. Data on WT and ΔG*znps1* strains were analyzed by ANOVA.

Evaluation of fertility when iron is supplied. For *C. heterostrophus*, MMNOS with or without Fe(III)EDTA was prepared. Before autoclaving, stock solutions of Fe(III)EDTA (10 mM in water) were added to MMNOS such that a final concentration of 250 or 500 μM was achieved. Fertility was evaluated as described above.

For *G. zeae*, CJ medium with or without Fe(III)EDTA or ferric citrate was prepared (23). Before autoclaving, stock solutions of Fe(III)EDTA (10 mM) or ferric citrate (10 mM) were added to CJ medium such that a final concentration of 125 or 250 μM was achieved in both cases. Crosses were set up as described above. Induction of sexual development was carried out with 2.5% Tween 60 with or without 125 μM Fe(III)EDTA or ferric citrate. Fertility was evaluated as described above.

Evaluation of fertility when ferricrocin is supplied. For *C. heterostrophus*, MMNOS with or without 200 μM ferricrocin was prepared. Ferricrocin (a generous gift from H. Haas, Innsbruck Medical University, Innsbruck, Austria) was applied to the medium after autoclaving. Crosses were set up by the standard method, and fertility was evaluated as described above.

For *G. zeae*, CJ medium with or without 100 μM ferricrocin was prepared. Crosses were set up as usual, except that induction of sexual development was carried out with 2.5% Tween 60 solutions with or without 100 μM ferricrocin. Fertility was evaluated as described above.

Expression analyses. Expression of *C. heterostrophus NPS2* and *G. zeae NPS1* and *NPS2* under iron-depleted conditions was examined as described previously (23). Briefly, total RNA was extracted from 48-h cultures grown in 30 ml MM or in MM without iron. Reverse transcription-PCR was carried out with SuperScript III (Invitrogen) by following the manufacturer's recommendations. cDNA samples were normalized on the basis of the expression of the gene that encodes *C. heterostrophus* glyceraldehyde phosphate dehydrogenase (*GPD1*) or *G. zeae* actin (*ACT1*). The primers used for this study are available on request.

Sensitivity to iron depletion. Sensitivity to iron depletion was examined as described previously (23), with the iron chelators 2,2′-dipyridyl (2DP) and bathophenathroline disulfonic acid (BPS). The MIC of the membrane-permeable iron chelator 2DP for each strain was determined by growing each strain on MM with twofold increasing concentrations of 2DP. Sensitivity to the membrane-impermeable iron chelator BPS was evaluated by comparing colony diameters of WT and Δ*nps2* strains (5 days old) on MM with or without 50 μM BPS.

Isolation, identification, and quantitative analysis of siderophores. Fungal cultures for HPLC analyses were prepared as described previously (23). Extraction of extracellular siderophores from culture broth was carried out as described by Oide et al. (23). For isolation of intracellular siderophores, freeze-dried mycelia were ground in approximately 40 ml of 15 mM potassium phosphate buffer (pH 7.5) with a pestle and a spatula and the suspension was subjected to sonication for 10 min. The insoluble fraction was removed by centrifugation, and the supernatant was collected. The insoluble fraction was resuspended in fresh potassium phosphate buffer (~40 ml) and subjected to sonication for 10 min. After centrifugation to remove the insoluble fraction, the soluble fractions (about 80 ml) were combined and subjected to siderophore extraction on XAD-16 resin as described previously (23).

For quantitative analysis of siderophore production, crude siderophore fractions were treated to solid-phase extraction as described previously (23), reconstituted in 200 μl of a 50:50 mixture of methanol and HPLC mobile components (50:50 acetonitrile–15 mM ammonium acetate, pH 4.5), filtered through a 0.4-μm-pore-size polyvinylidene difluoride membrane, and then analyzed by HPLC with an injection volume of 10 μl for broth samples and 20 μl for mycelial samples. Analytical HPLC was conducted on a Polaris C_{18} 5-μm column (250 by 4.6 mm) with a binary mobile phase consisting of acetonitrile (A) and 15 mM ammonium acetate buffer (pH 4.5) (B) with gradient elution at a flow rate of 1.0 ml/min (2.5% A for 1 min, a linear ramp up to 60% A in 13 min, 60% A for 2 min, and a linear ramp down to 2.5% A in 1 min). Detection was by absorption at 215 and 435 nm. Quantitative estimates of siderophore production were made

magnification. Images B, E, H, and K were taken at ×40 magnification. *C. heterostrophus* Δ*nps2* strains form pigmented and albino (arrows) WT pseudothecia as well as WT strains do, in both Δ*nps2* × WT and Δ*nps2* × Δ*nps2* crosses. This was true also for crosses of all other Δ*nps* strains to the WT. Note that WT beaks (double-headed arrows) are found at the top of pseudothecia formed by Δ*nps2* strains. (M) Average number of pseudothecia per square centimeter of leaf area. Error bars indicate 95% confidence intervals. No significant differences were observed in the number of pseudothecia among WT × WT, WT × Δ*nps2*, and Δ*nps2* × Δ*nps2* crosses ($P > 0.05$). (C, F, I, L) Microscope images of pseudothecial contents, taken at ×125 magnification. Pseudothecia from 21-day-old cross plates were crushed and stained with lactophenol-cotton blue. Mature asci and ascospores were found in pseudothecia from WT × WT and WT × Δ*nps2* crosses (C, F, I). In contrast, no asci and ascospores developed in pseudothecia formed in Δ*nps2* × Δ*nps2* crosses (L). (N) Average number of asci per pseudothecium. Error bars indicate 95% confidence intervals. Thirty pseudothecia were opened for each type of cross. No significant differences were observed in the number of asci per pseudothecium between WT × WT and WT × Δ*nps2* crosses ($P > 0.05$). In contrast, none of the 30 pseudothecia harvested from Δ*nps2* × Δ*nps2* crosses had mature asci or ascospores.

by HPLC after determination of standard curves. Limits of detection were established conventionally at a signal-to-noise ratio of 3.

Coprogen, neocoprogen I, and neocoprogen II, used as reference standards, were purified as described previously (23). Ferricrocin and triacetylfusarinine C (TAFC), used as reference standards, were purified as described by Oberegger et al. (22). Electrospray mass spectra were obtained as described previously (23).

RESULTS

Screening of the 12 *C. heterostrophus nps* deletion strains for a role in sexual development. When each of the 12 *C. heterostrophus* Δ*nps* strains (carrying individual deletions of *NPS1* to *NPS12*) was crossed to an albino WT strain of the opposite mating type, each resulting strain was fully fertile (data not shown). Both pigmented and albino pseudothecia formed, as in WT crosses, indicating that each Δ*nps* strain could act either male or female (compare Fig. 1A and B to D and E, data shown for the Δ*nps2* strain). No clear difference was observed in the average number of asci per pseudothecium of Δ*nps* crosses, compared to that of WT crosses (compare Fig. 1C with F). No significant reduction in the number of pseudothecia formed or in the number of asci per pseudothecium was observed, whether a Δ*nps* strain acted male (as evidenced by the presence and fertility of albino pseudothecia) or female (as evidenced by the presence and fertility of pigmented pseudothecia) (Fig. 1N, left three bars).

***C. heterostrophus* Δ*nps2* strains are defective in sexual development.** When each of the *C. heterostrophus* Δ*nps* strains with one of the 12 *NPS* genes deleted was homozygous in a cross, one type of cross (Δ*nps2* × Δ*nps2*) was sterile. When any of the other 11 *C. heterostrophus* Δ*nps* strains participated in a homozygous cross, the resulting strains were as fertile as in crosses to the WT (data not shown).

In the homozygous crosses with Δ*nps2* strains, WT numbers of both pigmented and albino pseudothecia were produced (Fig. 1J, K, and M, right bar); however, these were barren (Fig. 1L and N, right bar). No mature asci or ascospores developed in these pseudothecia, even when cross plates were incubated for 30 days after cross setup. WT crosses are generally mature by 21 days. Furthermore, results were the same when the deletion of the *NPS2* gene was in a *MAT1-1* background (compare Fig. 1G, H, and I to D, E, and F; middle bars in panel M; data not shown for ascus/ascospore development).

Reintroduction of the *C. heterostrophus* WT *NPS2* ORF into the Δ*nps2* strain restored WT fertility capability to the Δ*nps2* strain (data not shown).

***C. heterostrophus NPS2* encodes an NRPS responsible for ferricrocin biosynthesis.** Previous phylogenetic analyses indicated that *C. heterostrophus NPS2* is closely related to *NPS* genes involved in ferrichrome-type siderophore biosynthesis in other fungal species (i.e., *sidC* of *A. nidulans* or *sid2* of *U. maydis*) (18), suggesting that *NPS2* also plays a role in ferrichrome-type siderophore biosynthesis in *C. heterostrophus*. In contrast to *C. heterostrophus* Δ*nps6* strains, which are defective in extracellular siderophore biosynthesis (23), *C. heterostrophus* Δ*nps2* strains do not show any increase in sensitivity to iron depletion on media including the iron chelator 2DP or BPS, compared to WT strains (Fig. 2A and B). Nevertheless, expression of Ch*NPS2* was very weakly induced under iron-depleted conditions (Fig. 2C), which suggested to us that *C. heterostrophus NPS2* may have a role in iron metabolism.

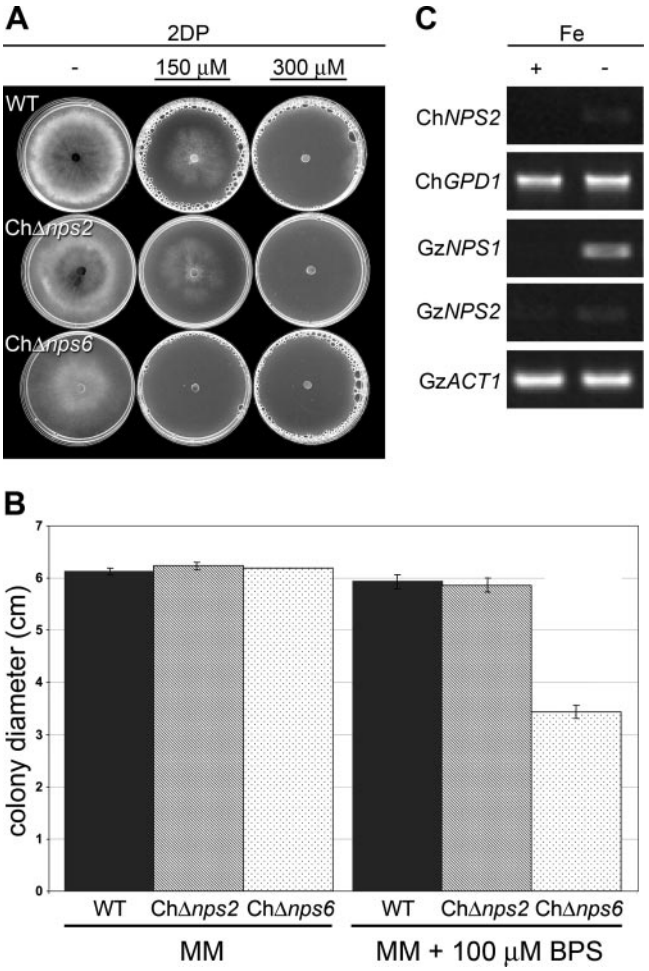


FIG. 2. Expression of *NPS2* is induced by iron depletion, but deletion of *NPS2* does not affect sensitivity to iron-depleted conditions. (A) The Δ*nps2* strain shows WT tolerance to the membrane-permeable iron chelator 2DP. Five-day-old cultures of *C. heterostrophus* WT, Δ*nps2*, and Δ*nps6* strains are shown. The MIC of 2DP for the WT and Δ*nps2* strains is 300 μM, while that for the Δ*nps6* strain is 150 μM. No significant difference in growth on MM with 150 μM 2DP was observed between the WT and Δ*nps2* strains, demonstrating that the Δ*nps2* strain is as tolerant to 2DP as the WT is. Note that some of the plates have bubbles in the agar at the edges. (B) The Δ*nps2* strain shows WT tolerance to the membrane-impermeable iron chelator BPS. The average colony diameter of 5-day-old cultures grown on MM with or without 100 μM BPS is shown for *C. heterostrophus* WT, Δ*nps2*, and Δ*nps6* strains. Three replicates were set up for each strain under each condition. Error bars indicate 95% confidence intervals. No significant difference in sensitivity to BPS was observed between the WT and Δ*nps2* strains. The Δ*nps6* strain shows increased sensitivity to BPS, as reported previously (23). (C) *C. heterostrophus NPS2* and *G. zeae NPS1* and *NPS2* are up-regulated under iron-depleted conditions. Expression of *C. heterostrophus NPS2* (Ch*NPS2*) and *G. zeae NPS1* and *NPS2* (Gz*NPS1* and Gz*NPS2*) in 48-h-old cultures grown under iron-replete (+) or iron-depleted (−) conditions is shown. Ch*NPS2*, Gz*NPS1*, and Gz*NPS2* are induced by iron depletion, while the expression levels of the controls, Ch*GPD1* and Gz*ACT1*, are approximately the same under both conditions.

HPLC analyses of *C. heterostrophus* WT and Δ*nps2* strains revealed that the Δ*nps2* strain lacks the ability to biosynthesize ferricrocin, the intracellular siderophore of *C. heterostrophus* (Fig. 3, peak 2). The *C. heterostrophus* Δ*nps2* strain, however,

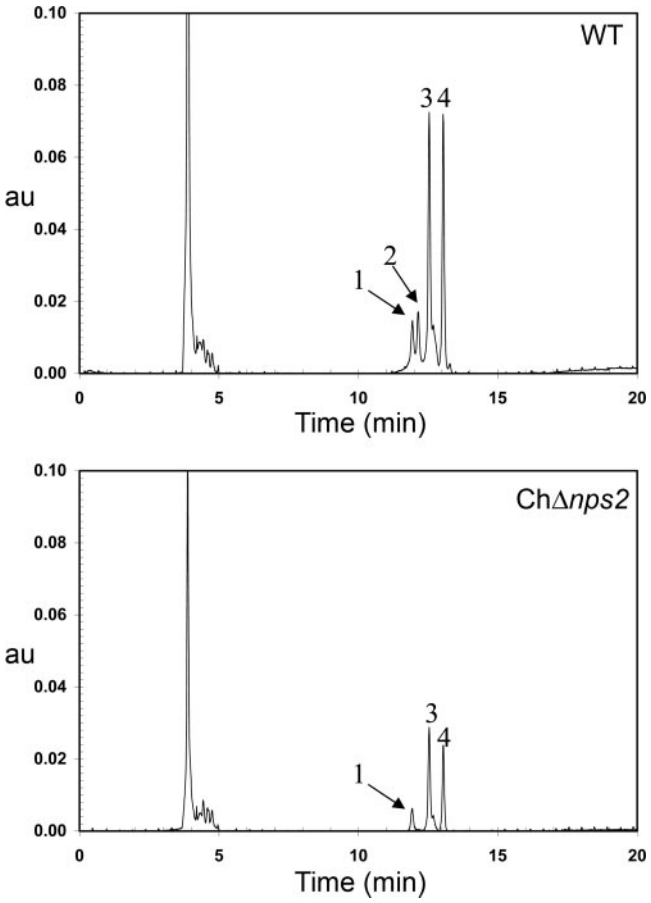


FIG. 3. The NRPS encoded by *NPS2* is responsible for intracellular siderophore biosynthesis in *C. heterostrophus*. HPLC analyses of mycelial extracts of WT (top) and Δ*nps2* (bottom) strains. Siderophore peaks are as follows: 1, neocoprogen II; 2, ferricrocin; 3, neocoprogen I; 4, coprogen. The ferricrocin peak is missing in the Δ*nps2* sample, demonstrating that the NRPS encoded by *NPS2* is responsible for ferricrocin biosynthesis. As observed previously (23), coprogen and its derivatives were detected in mycelial extracts, as well as in culture broth (not shown here). Note that coprogen and the derivatives were present in the Δ*nps2* sample, demonstrating that NRPS encoded by *NPS2* is not responsible for coprogen biosynthesis. au, absorbance units.

still produces extracellular siderophores (broth extract, data not shown) and their presence is detectable in mycelial extracts (Fig. 3, peaks 1, 3, and 4; see Fig. 5 in reference 23), demonstrating that the NRPS encoded by *C. heterostrophus NPS2* plays a role in intracellular siderophore biosynthesis but not in extracellular siderophore production.

Identification of the ortholog of *C. heterostrophus* NPS2 in *G. zeae*. Of the 12 *NPS* genes identified in *C. heterostrophus*, *NPS2* is one of the few conserved among diverse species of fungi (18). When the AMP-binding domains of *C. heterostrophus* NPS2, *A. nidulans* SidC, and *U. maydis* Sid2 were used as queries in a BLAST search against the *G. zeae* database (http://mips.gsf.de/genre/proj/fusarium/), two *G. zeae* candidates (FG05372.1/fgd457-680 and FG11026.1/fgd217-330) were identified as top matches. When the three AMP-binding domains from FG05372.1 and the three AMP-binding domains from FG11026.1 were added to the data set used to build the phylogenetic tree shown in Fig. 7 of reference 18, all six AMP domains grouped in the NPS2 siderophore clade (data not shown). FG05372.1 is designated *G. zeae NPS2*, and FG11026.1 is designated *G. zeae NPS1* (http://mips.gsf.de/genre/proj/fusarium/). Recent analyses of the *NPS2/1* siderophore clade indicate that there may have been a duplication of an ancestral gene and that Gz*NPS2* groups with the Ch*NPS2* lineage, while Gz*NPS1* groups with the *A. nidulans sidC* and *U. maydis sid2* lineage (Bushley and Turgeon, unpublished).

NPS2 function is conserved between heterothallic *C. heterostrophus* and homothallic *G. zeae*. To examine the functional conservation of NPS2, strains carrying a deletion of *G. zeae NPS2* were characterized with respect to sexual development in this unrelated homothallic ascomycete. Like *C. heterostrophus NPS2*, *G. zeae NPS2* was weakly up-regulated under iron-depleted conditions (Fig. 2C) and *G. zeae* Δ*nps2* strains showed WT tolerance to iron depletion (data not shown). As observed for Δ*nps2* strains of *C. heterostrophus*, *G. zeae* Δ*nps2* strains are defective in sexual development, in this case, in self-compatible sexual development. *G. zeae* Δ*nps2* strains form perithecia (Fig. 4D) as well as the selfed WT does (Fig. 4A), and there is no significant difference in the number of perithecia formed (Fig. 4C, left two bars). However, no mature asci or ascospores are found in these perithecia (Fig. 4E and F, middle bar), as for Δ*nps2* strains of *C. heterostrophus*.

HPLC analyses of the *G. zeae* WT strain identified ferricrocin as an intracellular siderophore of this species. HPLC analyses of the *G. zeae* Δ*nps2* strain revealed that it completely lacks ferricrocin in its cells, demonstrating that the NRPS encoded by *G. zeae NPS2* is responsible for the biosynthesis of ferricrocin (Fig. 5, compare the top panel [arrow] to the panel second from the top). Biosynthesis of TAFC, the extracellular siderophore of *G. zeae*, was observed in the broth extract of the Δ*nps2* strain (data not shown), indicating that the NRPS encoded by *G. zeae NPS2* does not play a role in TAFC biosynthesis. Quantitative analyses of TAFC biosynthesis indicated that the amount of TAFC produced was increased in Δ*nps2* strains (Fig. 6A and B, second bar from the right) compared to that in WT strains (Fig. 6A and B, left bar).

Deletion of *G. zeae NPS1*, which is closely related to *G. zeae NPS2*, does not affect sexual development. *G. zeae NPS1* is up-regulated under iron-depleted conditions (Fig. 2C), as is *G. zeae NPS2*, implying that the NRPS encoded by *G. zeae NPS1* is also involved in iron metabolism. Like *G. zeae* Δ*nps2* strains, *G. zeae* Δ*nps1* strains are as tolerant to iron depletion as the WT is (data not shown). In contrast to selfed strains of *G. zeae* Δ*nps2*, however, selfed strains of *G. zeae* Δ*nps1* show WT fertility, producing perithecia (compare Fig. 4A and G) with mature asci and ascospores (compare Fig. 4B and H). No clear reduction in the number of perithecia (see Fig. S1A in the supplemental material) or in the number of asci per perithecium (see Fig. S1B in the supplemental material) was observed, compared to the WT.

Biosynthesis of both intracellular (arrow, Fig. 5, second row from the bottom) and extracellular (not shown) siderophores was observed in *G. zeae* Δ*nps1* strains. Quantitative HPLC analyses of WT and Δ*nps1* strains of *G. zeae*, however, indicated that the amount of both extracellular (Fig. 6A and B, second bar from the left) and intracellular (Fig. 6C and D, right bar) siderophore made by the Δ*nps1* strain was reduced compared to that of the WT strain (Fig. 6A to D, left bar),

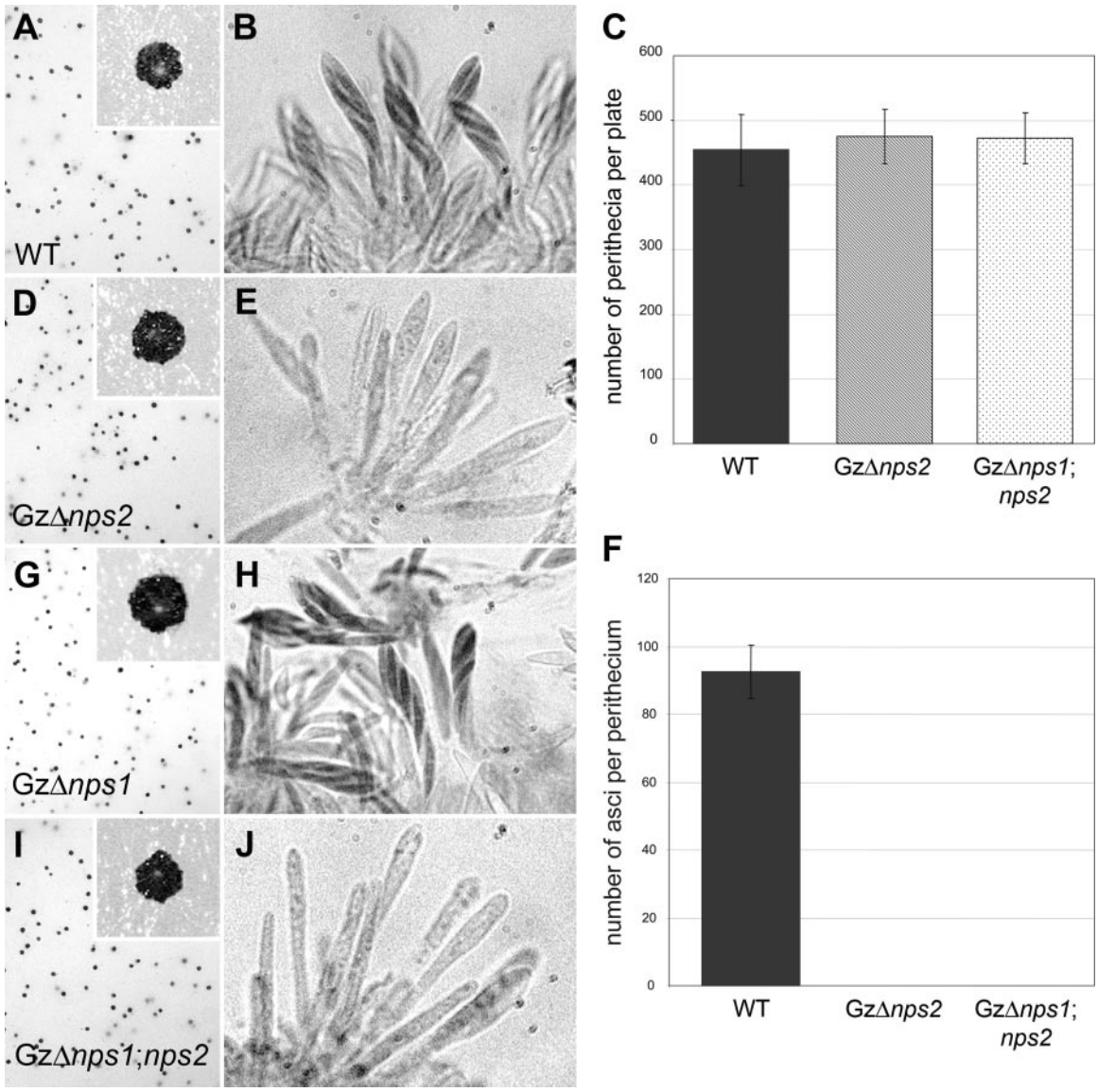


FIG. 4. *G. zeae* Δ*nps2* and Δ*nps1* Δ*nps2* strains form WT perithecia but fail to form mature asci and ascospores. *G. zeae* Δ*nps1* strains form perithecia with WT fertility. (A, D, G, I) Perithecia formed by WT, Δ*nps2*, Δ*nps1*, and Δ*nps1* Δ*nps2* selfed strains, taken at ×6.4 magnification, 14 days after cross setup; inserts, ×40 magnification. All strains form perithecia with WT morphology (compare panels D, G, and I with panel A). (C) Average number of perithecia per plate. Ten replicates were set up for each strain. Error bars indicate 95% confidence intervals. No significant differences were observed in the number of perithecia among the WT, Δ*nps2*, and Δ*nps1* Δ*nps2* strains. For data on Δ*nps1*, see Fig. S1 in the supplemental material. (B, E, H, J) Microscope images of the contents of perithecia, taken at ×500 magnification. Crushed perithecia harvested from 14-day-old crosses were stained with lactophenol-cotton blue. Abnormal ascus-like structures were observed in perithecia of Δ*nps2* and Δ*nps1* Δ*nps2* selfed strains (compare panels E and J with panel B). No ascospores were found in these ascus-like structures, in contrast to WT or Δ*nps1* asci. (F) Average number of asci per perithecium. Thirty perithecia were opened for each strain. No mature asci were found in perithecia developed in Δ*nps2* or Δ*nps1* Δ*nps2* selfed strains. Error bars indicate 95% confidence intervals. For data on Δ*nps1* fertility, which is like that of the WT, see Fig. S1 in the supplemental material.

suggesting that the NRPS encoded by *G. zeae NPS1* is somehow involved in siderophore biosynthesis.

The sexual development phenotype of strains carrying deletions of both *G. zeae NPS1* and *NPS2* is identical to that of *G. zeae* Δ*nps2* strains. Double deletion of *G. zeae NPS1* and *NPS2* does not affect tolerance to iron depletion (data not shown). The Δ*nps1* Δ*nps2* strain shows a defect in sexual development similar to that observed for Δ*nps2* strains; perithecia form as in the WT and Δ*nps2* strains (Fig. 4I and C, right bar; compare with panels A, D, and C, left two bars), but there are no mature asci or ascospores (Fig. 4J and F, right bar [absence of]; compare with panels E and F, middle bar [absence of]).

As expected, *G. zeae* Δ*nps1* Δ*nps2* strains completely lacked the ability to biosynthesize ferricrocin (Fig. 5, bottom row), while they were still able to biosynthesize extracellular siderophores (not shown). Quantitative HPLC analyses showed that the amount of extracellular (Fig. 6A and B, right bar) siderophore made by the Δ*nps1* Δ*nps2* strain was increased

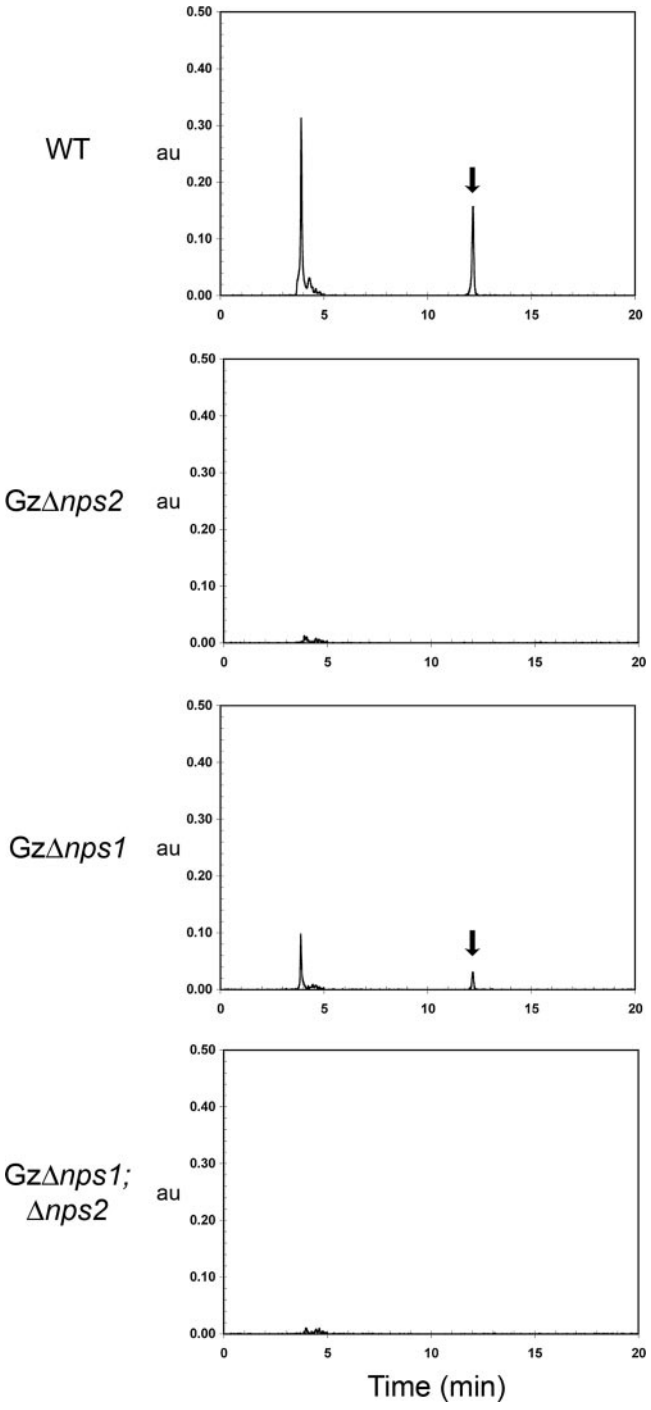


FIG. 5. The NRPS encoded by *G. zeae NPS2* is responsible for intracellular siderophore biosynthesis. HPLC analyses of mycelial extracts of WT, Δ*nps2*, Δ*nps1*, and Δ*nps1* Δ*nps2* strains are shown. Arrows indicate the ferricrocin peak. The ferricrocin peak is missing in the Δ*nps2* and Δ*nps1* Δ*nps2* samples (second panel from the top, bottom panel), demonstrating that the NRPS encoded by *NPS2* is responsible for ferricrocin biosynthesis. Biosynthesis of ferricrocin (and TAFC [not shown]) is observed in the Δ*nps1* sample (arrow, second panel from the bottom).

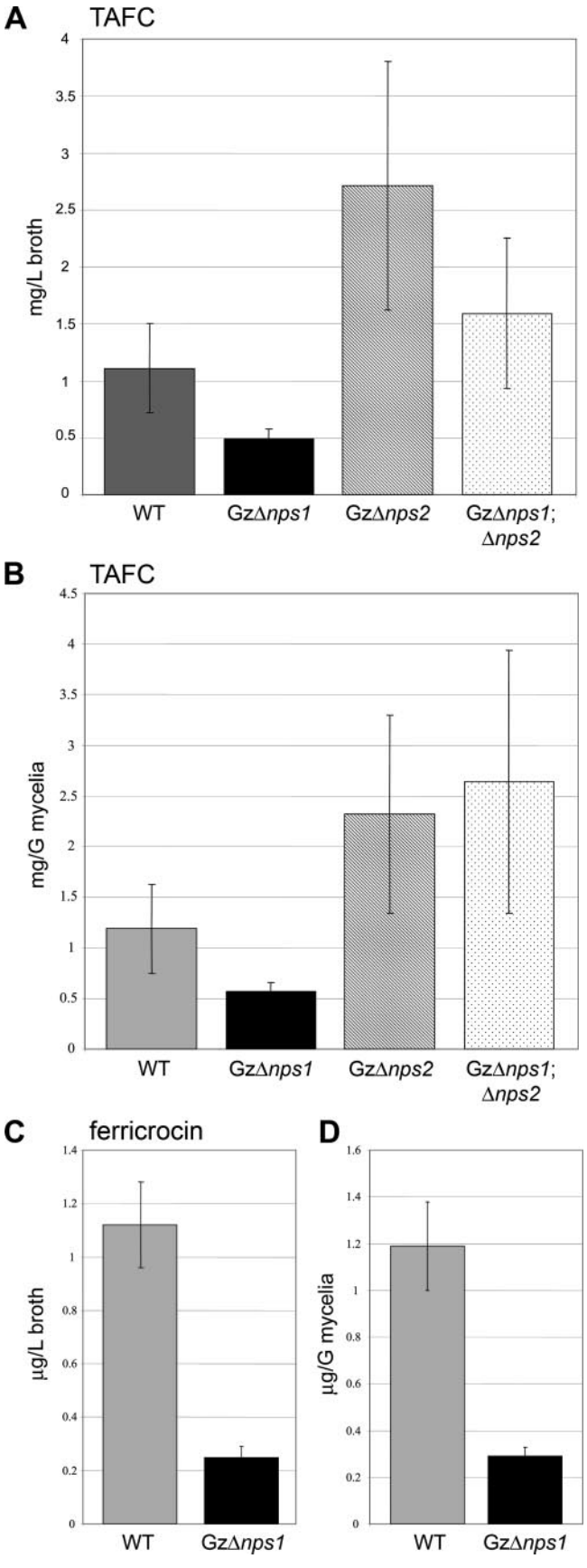
compared to that made by the WT or Δ*nps1* strain (Fig. 6A and B, compare the right bar to the first and second bars from the left). No significant difference in extracellular siderophore biosynthesis was observed between the Δ*nps1* Δ*nps2* and Δ*nps2* strains (Fig. 6A and B, compare the right bar to the second bar from the right).

Exogenous application of iron partially restores fertility to *C. heterostrophus* Δ*nps2* strains. The connection between the abnormal sexual development of *C. heterostrophus* Δ*nps2* strains and their lack of intracellular siderophore biosynthesis was further examined by characterizing the sexual development of Δ*nps2* strains supplied with iron during mating. Application of 250 μM Fe(III)EDTA partially restored fertility to *C. heterostrophus* Δ*nps2* strains (Fig. 7, compare panel B with panel D). *C. heterostrophus* Δ*nps2* strains form pseudothecia with some mature asci and ascospores, although the number of asci per pseudothecium is less than that of a WT cross (Fig. 7E; also, compare panel D with panels A and C). No significant difference in viability was observed between the ascospores recovered from these crosses and those from WT crosses (data not shown). Note that a reduction in the number of asci per pseudothecium was observed in WT crosses when iron was applied, indicating a deleterious effect of iron overload on the sexual reproduction of *C. heterostrophus* WT strains.

Exogenous application of iron partially or completely restores fertility to *G. zeae* Δ*nps2* and Δ*nps1* Δ*nps2* strains. Similarly, an exogenous supply of iron restores fertility to Δ*nps2* and Δ*nps1* Δ*nps2* strains of *G. zeae* (Fig. 8; see Fig. S2 in the supplemental material). On a plate supplied with 250 μM Fe(III)EDTA, both the *G. zeae* Δ*nps2* and Δ*nps1* Δ*nps2* strains formed perithecia with mature asci and ascospores (see Fig. S2E and F in the supplemental material; compare to Fig. 8B and C, respectively), although the average number of asci per perithecium was less than that of a WT strain (Fig. 8). Application of 250 μM ferric citrate, however, restored WT levels of fertility to *G. zeae* Δ*nps2* and Δ*nps1* Δ*nps2* strains (see Fig. S2H and I in the supplemental material; compare to Fig. 8B and C, respectively). No statistically significant difference ($P > 0.05$) was observed in the number of asci per perithecium among the WT, Δ*nps2*, and Δ*nps1* Δ*nps2* strains when ferric citrate was applied (Fig. 8). Thus, the *G. zeae* Δ*nps2* and Δ*nps1* Δ*nps2* strains show similar responses to an exogenous supply of iron, indicating that there is no significant difference between their defects in iron metabolism during sexual development.

Overall, these results obtained with *G. zeae* and the results obtained with *C. heterostrophus* Δ*nps2* strains demonstrate that the abnormal sexual development seen is at least partly due to their deficiency in nutrient iron resulting from loss of intracellular siderophores.

Exogenous application of ferricrocin restores WT fertility to Δ*nps2 strains*. Finally, we asked if application of ferricrocin, the intracellular siderophore of *C. heterostrophus* and *G. zeae*, is able to restore WT fertility to the Δ*nps2* strains. When ferricrocin was supplied, both the *C. heterostrophus* (Fig. 9) and *G. zeae* Δ*nps2* (Fig. 10; see Fig. S3 in the supplemental material) strains were as fertile as WT strains were. No significant differences in the number of asci per *C. heterostrophus* pseudothecium (Fig. 9E) or *G. zeae* perithecium (Fig. 10) were observed between the Δ*nps2* and WT strains, demonstrating that



the observed defect in the sexual development of Δ*nps2* strains is due to loss of the ability to biosynthesize ferricrocin.

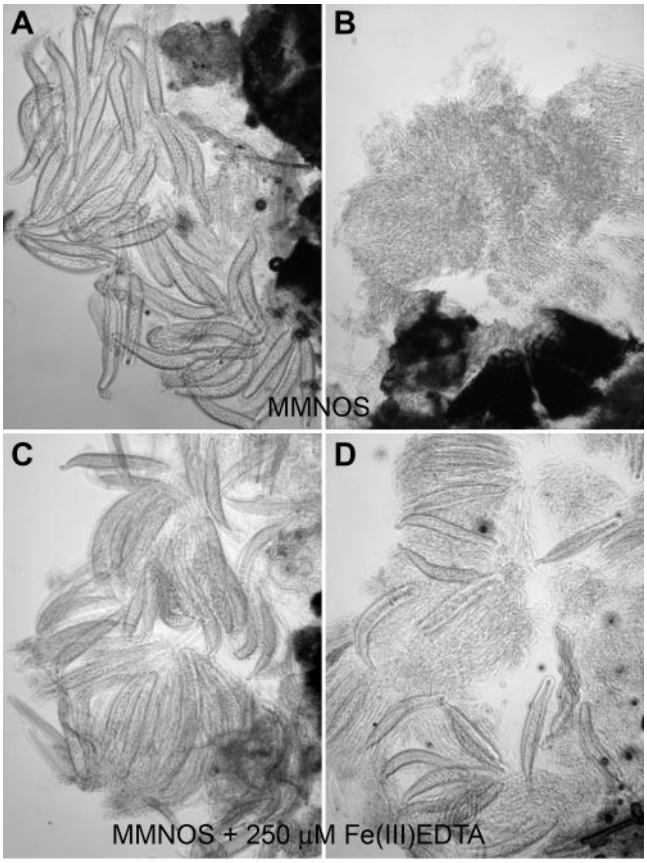
DISCUSSION

By screening our collection of Δ*nps* deletion strains, we have determined that a natural biological function of the intracellular siderophore ferricrocin, a metabolite biosynthesized by the NPS2 enzyme of the organisms that produce it, e.g., *C. heterostrophus* and *G. zeae*, is to participate in a fundamental fungal developmental process, i.e., production of sexual spores in meiosis. Thus, the observation that there is a close relationship between secondary-metabolite or small-molecule production and fungal development (7) is additionally documented. Furthermore, this observation provides support for the argument that products of multifunctional enzymes (e.g., NRPSs, best known as producers of secondary metabolites) can play fundamental roles in fungal cells themselves. The well-studied effects of these products on other organisms, either beneficial (e.g., as antibiotics) or harmful (e.g., as mycotoxins), may not address the primary "reason" why fungi and bacteria biosynthesize so-called secondary metabolites. The primary activities of these metabolites, one supposes, are those that benefit the fungal cell itself and not those that benefit pharmaceutical concerns.

Ferricrocin, the intracellular siderophore of *C. heterostrophus*, plays a role in sexual development. In this study, we characterized our collection of 12 Δ*nps* strains of *C. heterostrophus* with respect to a possible role in sexual development and found that the Δ*nps2* strains fail to develop asci in homozygous Δ*nps2* crosses. Phylogenetic (18) and expression analyses of *NPS2* indicated that the NRPS encoded by *NPS2* is involved in siderophore biosynthesis. MS and HPLC analyses demonstrated that the product of the NRPS encoded by *NPS2* is ferricrocin, the intracellular siderophore of *C. heterostrophus* (see Fig. 3 and 5 in reference 23). Exogenous application of iron restored the ability of *C. heterostrophus* Δ*nps2* strains to develop asci and ascospores, albeit at a reduced level. Exogenous application of ferricrocin itself fully restored WT fertility to the Δ*nps2* strains. To our knowledge, this is the first demonstration that iron and siderophores play a role in the sexual development of a heterothallic ascomycete fungus.

Nutrient iron stored by intracellular siderophores is essential for ascus and ascospore development in *C. heterostrophus*. Intracellular siderophores have been proposed to play a role in

FIG. 6. Biosynthesis of intra- and extracellular siderophores is reduced in the *G. zeae* Δ*nps1* strain, compared to that in the WT strain. (A, B) Amounts of TAFC biosynthesized per liter of broth (A) or per gram of mycelia (B) were compared among the WT, Δ*nps1*, Δ*nps2*, and Δ*nps1* Δ*nps2* strains of *G. zeae*. A statistically significant reduction in TAFC biosynthesis was observed in the Δ*nps1* strain, compared to the WT strain. The Δ*nps2* and Δ*nps1* Δ*nps2* strains produce more TAFC than the WT does, indicating that deletion of *NPS2* leads to an increase in the flow of precursor for siderophore biosynthesis to the NRPS encoded by *NPS6*. (C, D) Biosynthesis of ferricrocin per liter of broth (C) or per gram of mycelial extracts (D) was compared between WT and Δ*nps1* strains of *G. zeae*. A statistically significant reduction in ferricrocin biosynthesis was observed in the Δ*nps1* strain, compared to the WT strain. The Δ*nps2* and Δ*nps1* Δ*nps2* strains do not make ferricrocin.



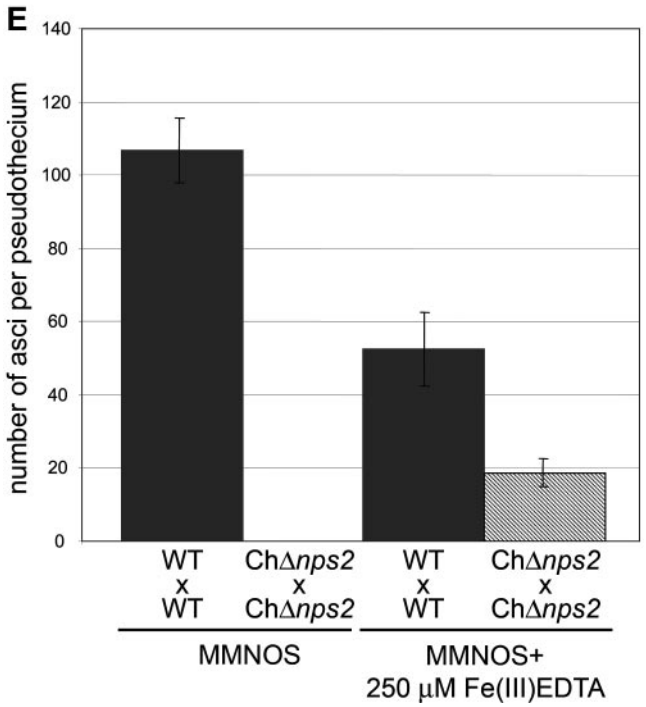


FIG. 7. Exogenous application of iron partially restores fertility to *C. heterostrophus* Δ*nps2* strains. (A to D) Microscope images of the contents of pseudothecia, taken at ×125 magnification. Crushed pseudothecia harvested from 21-day-old cross plates were stained with lactophenol-cotton blue. Mature asci and ascospores were found in pseudothecia developed in Δ*nps2* × Δ*nps2* crosses on plates containing 250 μM Fe(III)EDTA (D). (E) Average number of asci per pseudothecium. Thirty pseudothecia were opened for each strain and for each condition. Error bars indicate 95% confidence intervals. With a supply of 250 μM Fe(III)EDTA, Δ*nps2* × Δ*nps2* crosses were fertile, although the number of asci per pseudothecium was less than that of WT crosses. Note that a statistically significant reduction in fertility was observed in the WT cross when iron was applied, compared to this cross under standard conditions (MMNOS), suggesting possible deleterious effects of iron overload.

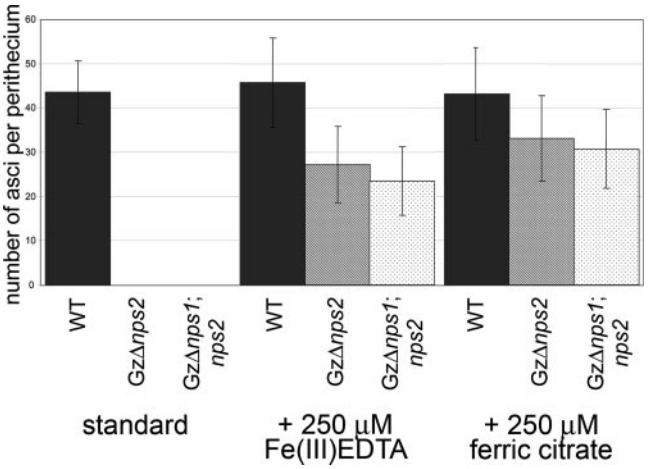
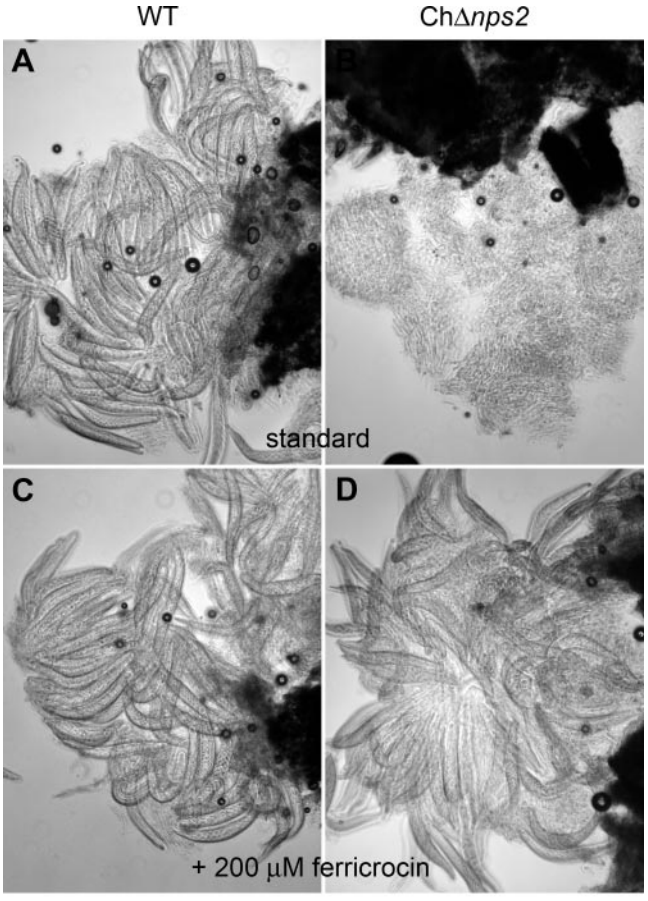


FIG. 8. Application of iron restores WT fertility to *G. zeae* Δ*nps2* and Δ*nps1* Δ*nps2* strains. The average number of asci per perithecium is shown. Thirty perithecia were opened for each strain and for each condition. Error bars indicate 95% confidence intervals. When 250 μM ferric citrate was supplied, no significant differences were observed in the number of asci per perithecium among WT, Δ*nps2*, and Δ*nps1* Δ*nps2* selfed strains, demonstrating that application of ferric citrate restores WT fertility to Δ*nps2* and Δ*nps1* Δ*nps2* strains.

iron storage in fungi (36). In this study, exogenous application of iron in the form of Fe(III)EDTA partially restored the ability of *C. heterostrophus* Δ*nps2* strains to develop asci and ascospores, indicating that their defect in sexual development is at least partly due to loss of the ability to store iron and thus is due to starvation for nutrient iron. Whether a Δ*nps2* strain acts male or female makes no difference in crosses between WT and Δ*nps2* strains; these crosses are fully fertile, demonstrating that the stored iron supply in one of the two mating partners is sufficient for successful sexual development and that the mating type has no connection to the sexual developmental phenotype of Δ*nps2* strains. In WT × Δ*nps2* crosses, iron bound to intracellular siderophores is accessible to the Δ*nps2* mating partner presumably only after cell-cell fusion with a WT mating partner; thus, nutrient iron stored by intracellular siderophores must be relatively unimportant for development prior to cell-cell fusion.

Furthermore, *C. heterostrophus* Δ*nps2* strains form WT pseudothecia even in homozygous Δ*nps2* crosses, demonstrating that absence of iron storage capability by siderophores does not affect pseudothecium development. The block in the sexual development of *C. heterostrophus* Δ*nps2* strains appears to be before initiation of ascus formation, although rare immature ascus-like structures can be found in pseudothecia of homozy-



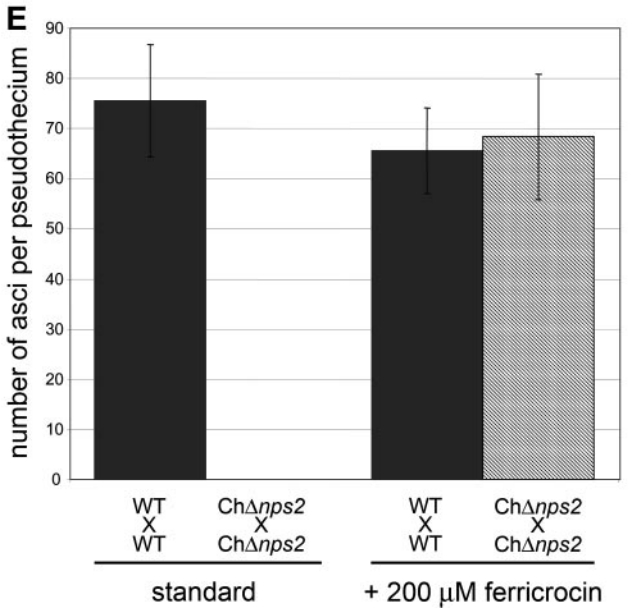


FIG. 9. Application of ferricrocin restores WT fertility in homozygous *C. heterostrophus* Δ*nps2* crosses. (A to D) Microscope images of the contents of crushed pseudothecia, taken at ×125 magnification, collected from 21-day-old cross plates stained with lactophenol-cotton blue. On plates containing 200 μM ferricrocin, Δ*nps2* × Δ*nps2* crosses developed pseudothecia with mature asci and ascospores (D). (E) Average number of asci per pseudothecium. Thirty pseudothecia were opened for each strain and for each condition. Error bars indicate 95% confidence intervals. When ferricrocin was supplied, no significant differences in the number of asci per pseudothecium were observed between Δ*nps2* × Δ*nps2* and WT × WT crosses ($P > 0.05$), demonstrating that ferricrocin restores WT fertility to Δ*nps2* strains. The standard was MMNOS.

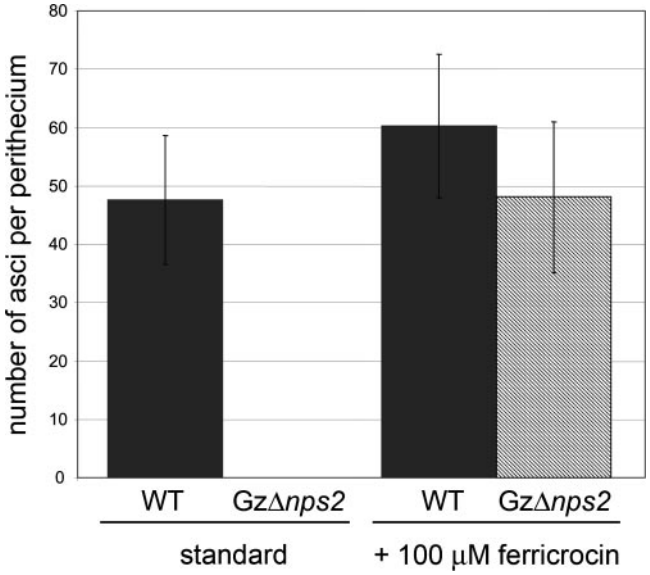


FIG. 10. Application of ferricrocin restores WT fertility to *G. zeae* Δ*nps2* selfed strains. The average number of asci per perithecium is shown. Error bars indicate 95% confidence intervals. When ferricrocin was applied, no significant differences in the number of asci per perithecium were observed between Δ*nps2* and WT selfed strains ($P > 0.05$), demonstrating that ferricrocin restores WT fertility to the Δ*nps2* strain. The standard was CJ medium.

gous Δ*nps2* crosses (not shown). In summary, nutrient iron stored by intracellular siderophores is essential for ascus/ascospore development in *C. heterostrophus* but dispensable for early sexual development, including pseudothecium formation.

Extracellular and intracellular siderophores play fundamentally different roles in fungal developmental biology. Inhibition of extracellular siderophore biosynthesis through deletion of *NPS6* leads to a reduction of pigmentation and asexual sporulation in *C. heterostrophus* (23). By contrast, Δ*nps2* strains show WT pigmentation and WT levels of asexual sporulation (data not shown), indicating that a major source of iron during asexual development of *C. heterostrophus* is iron influx through extracellular siderophores, rather than iron stored by intracellular siderophores. Extracellular siderophores are required for full virulence of *C. heterostrophus* to its host, while intracellular siderophores are dispensable for fungal infection, as evidenced by the WT virulence of the Δ*nps2* strain to the host. Thus, iron acquired by extracellular siderophores is a major source of iron also in planta. Extracellular siderophores are apparently dispensable for sexual development, since Δ*nps6* strains show WT fertility both in heterozygous and in homozygous Δ*nps6* crosses, indicating that iron stored in intracellular siderophores is more important for iron metabolism of *C. heterostrophus* during sexual development. On the basis of the data presented here and our previous work on extracellular siderophores of *C.*

heterostrophus (23), we propose that intra- and extracellular siderophores play fundamentally different roles in *C. heterostrophus*.

Intracellular siderophores, reactive oxygen species (ROS), and fungal sexual development. In addition to their role in iron storage, fungal intracellular siderophores are proposed to play a role also in sequestering iron to prevent Haber-Weiss/Fenton reactions, which generate highly cytotoxic hydroxyl radicals (22). The Haber-Weiss/Fenton reaction, summarized as $O_2\cdot^- + H_2O_2 \rightarrow O_2 + OH\cdot + OH^-$, is catalyzed by intracellular free iron (14). To avoid this cytotoxic reaction, many organisms sequester intracellular iron in iron-binding proteins, such as transferrin and ferritin (16, 24). The antioxidant property of ferritins, through sequestration of iron, has been well characterized in bacteria, plants, and animals (3, 30). Ferritins, if present in fungi, are largely uncharacterized. A ferritin-like protein in a fungus was reported for the first time only recently (29). In fungal cells, the cytoprotective role that ferritin plays in other organisms (against hydroxy radicals produced by Haber-Weiss/Fenton reactions) is proposed for intracellular siderophores (22).

In *A. nidulans*, deletion of *sidC*, which encodes the NRPS responsible for intracellular siderophore biosynthesis, leads to an increase in intracellular free-iron levels and to hypersensitivity of mycelia to the redox-cycling agent paraquat (12). Asexual spores of an *A. nidulans* Δ*sidC* strain show hypersensitivity to ROS more clearly than mycelia (13). In our experiments, no significant difference in sensitivity to ROS was observed between WT and Δ*nps2* strains of *C. heterostrophus* during vegetative growth (see Fig. S4 in the supplemental material). Likewise, a mutant strain of *Schizosaccharomyces pombe* which lacks the ability to biosynthesize intracellular siderophores is as tolerant to paraquat as is the WT (28). The role of intracellular siderophores as an antioxidant may be more important in fungal spores than in mycelia, at least in some fungi.

Sensitivity to iron overload stress was also examined in *C. heterostrophus*, but no clear difference was observed between the WT and Δ*nps2* strains (data not shown). Nevertheless, exogenous application of iron led to reduced fertility in a homozygous WT cross of *C. heterostrophus*, suggesting that excess iron does have adverse effects on the sexual development of this fungus. It is noteworthy that exogenous application of iron bound to ferricrocin did not cause a reduction of fertility in a homozygous WT cross of *C. heterostrophus*, indirectly supporting the hypothesis of the cytoprotective role of intracellular siderophores. Exogenous application of iron only partially restored fertility to *C. heterostrophus* Δ*nps2* strains in a homozygous Δ*nps2* cross, while application of ferricrocin fully restored fertility. A simple interpretation of these results is that iron was not as efficiently taken up or distributed by fungal cells as was ferricrocin. Alternatively, the results could imply that there is an as-yet-unknown function of intracellular siderophores in fungal sexual development, in addition to their role as a source of nutrient iron. Recent studies imply a role for ROS in fungal development (reviewed in reference 1). For example, expression of *cpeA*, which encodes a catalase-peroxidase in *A. nidulans*, is induced during sexual development (26). Perhaps fungal intracellular siderophores play an antioxidant role in sexual development, in addition to their role as a source of nutrient iron.

The roles of NPS2 in ferricrocin biosynthesis and in sexual development are conserved between the heterothallic ascomycete *C. heterostrophus* and the homothallic ascomycete *G. zeae*. To date, all of the known fungal siderophores are of the hydroxamate type (34), except for polycarboxylate siderophores produced by zygomycetes (31). Hydroxamate siderophores are largely divided into three groups, the ferrichrome type, the coprogen type, and the fusarinine type (25). Our previous study identified the gene (*NPS6*) that encodes an NRPS responsible for the biosynthesis of coprogen and its derivatives in *C. heterostrophus* (23). *NPS6* is one of only a few *NPS* genes conserved among diverse species of fungi (18). We demonstrated that the NRPSs encoded by *NPS6* orthologs are responsible for the biosynthesis of coprogen- and fusarinine-type extracellular siderophores in *A. brassicicola* and *G. zeae*, respectively, and proposed the functional conservation of NPS6 in coprogen- or fusarinine-type siderophore biosynthesis among filamentous ascomycetes (23). In the present study, we revealed that the NRPS encoded by *C. heterostrophus NPS2* is responsible for biosynthesis of ferricrocin, a ferrichrome-type siderophore. *NPS2* is also conserved among diverse species of fungi, including ascomycetes and basidiomycetes (18). The present study and an independent study by Tobiasen et al. (32) identified ferricrocin as the product of the NRPS encoded by *G. zeae NPS2*. *sidC* and *sid2*, to which *NPS2* genes show strong similarity, encode the NRPSs responsible for the biosynthesis of ferricrocin and ferrichrome in *A. nidulans* and *U. maydis*, respectively (12, 38). Schwecke et al. (28) demonstrated that the NRPS encoded by the *NPS2* ortholog of *S. pombe* (*sib1*) is responsible for ferrichrome biosynthesis. On the basis of in silico analyses, they also predicted that the NRPSs encoded by *NPS2* homologs are likely to produce similar products in different species of fungi. Taking the findings together, we propose that the function of the NRPS encoded by *NPS2* in ferrichrome-type siderophore biosynthesis is conserved among diverse species of fungi.

Our study on *NPS6* demonstrated that the role of extracellular siderophores in fungal virulence to plant hosts is conserved among filamentous ascomycetes (23). Here, we demonstrated that the role of intracellular siderophores in sexual development is conserved in the heterothallic ascomycete *C. heterostrophus* and the homothallic ascomycete *G. zeae*. Deletion of the *NPS2* ortholog in *G. zeae* resulted in concomitant defects in intracellular siderophore biosynthesis and ascus/ascospore development, as observed in *C. heterostrophus* Δ*nps2* strains. Application of ferricrocin fully restored WT fertility to the *G. zeae* Δ*nps2* strain. Recently, Eisendle et al. (13) reported that a Δ*sidC* strain is impaired in sexual development in the homothallic ascomycete *A. nidulans*. Application of ferricrocin restored the ability of the Δ*sidC* strain to undergo WT sexual development. Whether or not intracellular siderophores play a role in sexual development also in other species, such as the basidiomycete *U. maydis* and the fission yeast *S. pombe*, is the subject of future studies.

Gz*NPS1* has a potential role in the siderophore biosynthesis of *G. zeae*. Like *G. zeae NPS2*, *G. zeae NPS1* shows high similarity to *A. nidulans sidC* and *U. maydis sid2* and groups with these genes and *C. heterostrophus NPS2* in phylogenetic anal-

yses, suggesting a role in ferrichrome-type siderophore biosynthesis. Tobiasen et al. (32) proposed also that *G. zeae NPS1* is likely to encode the NRPS involved in siderophore biosynthesis, although no functional data were provided. Indeed, we found that *G. zeae NPS1* is strongly up-regulated under iron-depleted conditions, further indicating its involvement in iron metabolism. In this study, however, we failed to obtain clear evidence that the NRPS encoded by *G. zeae NPS1* has a role in siderophore biosynthesis. Deletion of *NPS6* in *G. zeae* abolished extracellular siderophore biosynthesis (23), and deletion of *G. zeae NPS2* led to complete loss of intracellular siderophore biosynthesis, demonstrating that the NRPSs encoded by *NPS6* and *NPS2* fully account for siderophore biosynthesis in *G. zeae*. Consistently, HPLC analyses of *G. zeae* Δ*nps1* strains confirmed the biosynthesis of ferricrocin and TAFC, the major intracellular and extracellular siderophores of *G. zeae*. Furthermore, any characteristic phenotype (sensitivity to low-iron and oxidative stress) associated with iron was not observed in *G. zeae* Δ*nps1* strains and the phenotype of *G. zeae* Δ*nps1* Δ*nps2* strains is identical to that of Δ*nps2* strains.

Nevertheless, quantitative HPLC analyses revealed an interesting difference in the levels of siderophore biosynthesis between WT and Δ*nps1* strains of *G. zeae*. When the amounts of siderophore biosynthesized per liter of culture broth, or per gram of mycelia, were compared, the amount of extracellular siderophore produced by *G. zeae* Δ*nps1* strains was approximately half of the amount produced by WT strains (Fig. 6A and B). Similarly, *G. zeae* Δ*nps1* strains produced only a quarter of the amount of intracellular siderophore produced by WT strains (Fig. 6C and D). Ferricrocin and TAFC are both trihydroxamate siderophores, and the proposed role of the NRPSs encoded by *NPS2* and *NPS6* is to conjugate 3 units of N^5-acyl-N^5-hydroxy-L-ornithine (AHO), the direct precursor of fungal hydroxamate siderophores. Perhaps the NRPS encoded by *NPS1* is partially functional and able to biosynthesize dihydroxamates but not trihydroxamates. These dihydroxamates may then be used by the NRPSs encoded by *NPS2* and *NPS6* to biosynthesize trihydroxamate siderophores via incorporation of a single AHO unit. Thus, perhaps the contribution of the NRPS encoded by *NPS1* may be in achieving efficient siderophore biosynthesis. This hypothesis can be tested by examining the biosynthesis of dihydroxamates in Δ*nps2* Δ*nps6* and Δ*nps1* Δ*nps2* Δ*nps6* strains of *G. zeae*. If the hypothesis is supported, production of dihydroxamates should be detected only in the former. Alternatively, the function of *G. zeae NPS1* could be examined in the *C. heterostrophus* Δ*nps2* strain by separately expressing the heterologous *G. zeae NPS1* and *NPS2* genes to see if they both complement (i.e., restore the WT phenotype to the *C. heterostrophus* Δ*nps2* strain), which would indicate functional redundancy.

Quantitative HPLC analyses also pointed out that Δ*nps2* and Δ*nps1* Δ*nps2* strains produce more TAFC than WT *G. zeae* strains (Fig. 6). A similar observation was made in the quantitative analyses of siderophore biosynthesis in WT and Δ*nps6* strains of *C. heterostrophus*. In this case, *C. heterostrophus* Δ*nps6* strains produced more intracellular siderophores than WT strains did (23; see Table S3 in the supplemental material). These results are due, most likely, to a change in the flow of AHO, the common precursor of intra- and extracellular siderophores. In the *G. zeae* Δ*nps2* strain, inhibition of the pathway for intracellular siderophore production probably led to an increase in AHO flow to the NRPS encoded by *NPS6*, resulting in an increase in TAFC biosynthesis. These results indicate that deletion of an *NPS* gene affects the biosynthesis of a metabolite which is not the product of the NRPS encoded by the *NPS* gene. Thus, reduction of siderophore biosynthesis in *G. zeae* Δ*nps1* strains could be due to secondary effects of deletion of *NPS1*.

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